Neuroprotective effects of Rg3-enriched Korean Red Ginseng on alcohol-induced apoptosis in PC12 Cells

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PC12 세포에서 알코올 유발성 세포 사멸에 대한 Rg3 풍부 고려 홍삼의 신경세포 보호 효과

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Abstract Excessive alcohol consumption is one of the leading causes of many neurological diseases, such as dementia and Alzheimer's disease, and many efforts are under way to solve them. Red ginseng is known to enhance neuronal survival, inhibit apoptosis, and promote nerve regeneration of nerve cells. This study examined whether Rg3-enriched Korean red ginseng extract (KRG) inhibits the apoptosis of PC12 cells caused by alcohol-induced neurotoxicity and how KRG regulates several factors related to the caspase mediated pathway. In this way, the cell survival rate and apoptosis rate of PC12 cells were measured using an EZ-Cytox cell viability assay kit and flow cytometry, respectively. The expression of the apoptosis-related proteins (Bcl-2, Bid, Bax and caspase-3) were analyzed by western blotting, and the significance of the measured results was confirmed using the ANOVA method. As a result, KRG increased the expression of Bcl-2; inhibited the expression of Bid, Bax, and caspase-3; and inhibited the apoptosis of alcohol-induced PC12 cells. These results mean that the KRG-induced increase in Bcl-2 expression and down-regulation of Bid and Bax expression down-regulate caspase-3 expression, which in turn inhibits the mitochondrial apoptotic pathways. This study suggests that KRG is worth developing as a neuroprotective agent candidate.

요 약 과도한 음주는 치매 및 알츠하이머 병과 같은 여러 신경계 질환을 일으키는 주요원인 중 하나로 알려져 있으며 이를 해결하기 위한 많은 노력이 진행 중이다. 또한, 홍삼은 신경 세포의 생존, 세포 자멸사의 억제 및 신경 세포의 신경 재생을 향상시키는 것으로 알려져 있다. 본 연구의 목적은 Rg3 풍부 고려홍삼 추출액 (KRG)이 알코올 유발성 신경독성으로 인하여 일어나는 PC12 세포의 세포 사멸을 억제 할 수 있는지, 그리고 KRG가 caspase 매개 경로와 관련된 몇 가지 인자들을 어떻게 조절하는지 확인하는 것이다. 그 방법으로, 우리는 PC12 세포에서의 세포 생존율과 세포 사멸율을 EZ-Cytox 세포 생존율 측정 kit와 유세포 분석기로 측정하였고, 세포 자멸 관련 단백질 (Bcl-2, Bid, Bax, caspase-3)의 발현 정도를 Western blot기법으로 측정하였으며, 측정된 결과의 유의성을 ANOVA 분석법으로 확인하였다. 그 결과, KRG는 Bcl-2의 발현을 증가시키고, Bid와 Bax 및 caspase-3 발현을 저해하였고, 이를 통해 알코올로 유도된 PC12 세포의 세포 사멸을 억제하였다. 이러한 결과를 통해, KRG에 의해 유도된 Bcl-2 발현의 증가와 Bid 및 Bax 발현의 하향 조절이 caspase-3 발현을 하향 조절하고, 결국 미토콘 드리아 세포 사멸 경로를 억제한다는 것을 결론내릴 수 있었다. 본 연구는 향 후, KRG가 신경 보호제 후보로서 개발할 가치 가 있음을 제시하였다.

1. Introduction

Alzheimer's disease (AD) is the common type of dementia, which is characterized as a progressive memory loss and some detrimental cognitive changes [1]. AD has multiple etiological reasons such as genetic, lifestyle, and environmental factors [1]. Excessive alcohol consumption can be one of main reasons causing several neurological disorders such as dementia and Alzheimer's disease [2]. In particular, many studies have shown the association of alcohol consumption and dementia [3-5]. Although the exact pathway is till not known, there are some evidences that the brains of alcoholics lead to toxic changes, including brain volume reduction [6-8]. Recent research has shown that one of many changes by alcohol-induced neurodegeneration is apoptosis [9-11]. In addition, chronic alcohol consumption leads to brain atrophy with general points of predilection for neurotoxic effects [12], and caspase-mediated apoptosis have been known to important mediators of cell death in some neurodegenerative disorders [13].

Korean Red Ginseng, the streamed root of Panax ginseng Meyer, have been used frequently as a traditional oriental medicine, and is known to include various pharmacological activities with anti- inflammatory action, antitumor, anti-diabetes, anti- oxidation, and anti-hepatotoxicity [14,15]. In addition, ginseng has been known to neuronal cell survival, to suppress apoptosis, and to enhance neuronal neuroregeneration [16]. In recent, it is reported that Korean Red Ginseng suppresses 1-methyl-4-phenylpyrinium-induced apoptosis in PC12 cell by increasing caspase-3 and caspase‐9 mRNA levels [17].

This present study is designed to investigate whether Rg3-enriched Korean Red Ginseng (KRG) can inhibit apoptosis of PC12 cell from alcohol-induced neurotoxicity, and how KRG regulate some factors concerned with caspase-mediated pathway more carefully. Therefore, we measured cell viability, cell apoptosis rate, and levels of the apoptosis-related proteins (Bcl-2, Bid, Bax and caspase-3) expression in PC12 cell. The purpose is to clarify the protective effect of KRG on alcohol- induced apoptosis in PC12 cell, which might provide a theoretical basis of KRG for a clinical treatment for alcohol-induced nerve injury.

2. Materials and methods

2.1 Materials

Rg3-enriched Korean red ginseng (KRG) were donated from Central Institutes of KT&G (Daejeon, Korea). **As** shown in Table 1, KRG mainly contained 42.04 mg/g (4.2%) on the 20(S)-Rg3. Anti- presenilin1, anti-Bcl-2, anti-caspase-3, anti-Bid, anti-Bax, β-actin, anti-rabbit IgG-horse radish peroxidase conjugate (HRP) antibodies, and lysis buffer were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyvinylidene difluoride (PVDF) membrane, Enhanced chemiluminescence (ECL) solution was bought from GE health care (Chalfont St. Giles, Buckinghamshire, UK). Rat adrenal phaeochromocytoma cells (PC12) were obtained from the American Type Culture Collection (Manassas, VA, USA). DMEM were purchased from Hyclone (Logan, UT, USA). Penicilin (100 U/ml), Streptomycin (100 µg/ml) and Fetal bovine serum (FBS) were purchased from Gibco (Life technology lnc., Gaithersburg, MD, USA). Cell viability was measured using EZ cytox Cell Viability Asaay Kit (Daeil Labservice, Seoul, Korea). To measure PC12 cell apoptosis, an Alexa FluorTM 488 Annexin V/Dead cell apoptosis Kit (Invitrogen, Eugene, Oregon) was used.

2.2 Methods
2.2.1 Cell culture

PC12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, 0.1% penicillin-streptomycin at 37°C under humidified atmosphere containing 5% CO_2.

Table 1. Ginsenoisde contents of KRG

	Ginsenoside content (mg/g)
Rg1	0.30
Rb1	4.14
20(S)-Rg3	42.04
Re	0.21
Rc	1.14
Rb2	1.62
Rd	1.70
Rf	1.47
Rh1	3.11
20(S)-Rg2	3.90
20(S)-Rg3	7.43

2.2.2 Cell viability analysis

Cell viability was measured using the EZ-Cytox Cell Viability Assay kit (Dogen, Seoul, Korea). The cells were seeded into 96-well plates (1×10^4 cells/ml), and cultures overnight. Next, cells were treated without or with various concentration of KRG. After 6 hr of incubation, cell were treated without or with 5% alcohol. After 24 hr of incubation, 10 μl of Ez-Cytox reagent was added to each well, and the cells were incubated for 30 min. The plates were analyzed with an enzyme-linked immunosorbent assay microplate reader (Molecular Device, Sunnyvale, CA, USA) at 450 nm.

2.2.3 Western blot analysis

Cell lysates were centrifuged 10,000 g at 4°C for 10 min and the supernatant containing the same protein (10 μg) were mixed with sample buffer and boiled for 5 min. Protein concentrations were measured by Quick Start Bradford Protein Assay Kit (Bio-rad). Proteins were isolated by 8% or 15% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes (Merk Millipore Ltd). Non-specific binding was blocked by 5% skim milk in TBST at room temperature for 1 hr. The membranes were incubated with primary antibody. After incubation with secondary antibodies, the membranes were visualized with ECL. The blots were analyzed by using Fusion SL imaging systems (Vilber Lourmat, France). The dilutions for anti-Presenilin1, anti-Bcl-2, anti-caspase-3, anti-Bid, anti-Bax and anti-rabbit IgG-horse radish peroxidase conjugate (HRP) antibodies were 1:2000.

2.2.4 Detection of apoptosis

The cells were seeded into 6-well plates (5×10^5 cells/ml), and cultured overnight. Then, cells were treated without (control) or with various concentrations of KRG. After 6 hr incubation, cell were treated without (control) or with 5% alcohol. After 24 hr incubation, the cells were washed using PBS buffer. An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Invitrogen) was used to detect apoptosis of PC12 cell. Cells were incubated with an FITC-labeled annexin V and propidium iodide (PI) at room temperature for 15 min, and then apoptotic PC12 cells were analyzed using NovoCyte Flow Cytometer (ACEA Biosciences).

2.2.5 Statistical analysis

The experimental results are expressed as the mean ± S.D., and statistical analysis was performed by ANOVA. If the analysis indicated significant differences among the group means, each group was compared by the Scheffe's method, and a p value<0.05 were considered significant.

3. Results

3.1 Effects of KRG on alcohol−treated PC12 cell viability

Alcohol induces neuron cell death in concentration-dependent manner (Fig. 1A). Therefore, reducing the neuron cell death by KRG might be protective against alcohol-induced neurotoxicity. When PC12 cell were exposed to 5% alcohol, cell survival rate was decreased to 73% over the control (100%), whereas KRG enhanced cell viability up to 112% at 1 μg/ml (Fig. 1B). These results indicated that PC12 cell death is suppressed by KRG in a concentration-dependent manner. Further, KRG is confirmed non-cytotoxic effect in a dose-dependent manner (Fig. 1C).
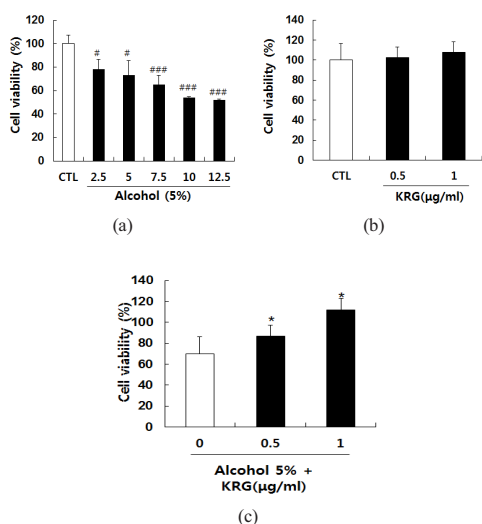
(a)

(b)

(c)

Fig. 1. Effect of KRG on alcohol-treated PC12 cell viability. (a) Effects of alcohol only on PC12 cell. (b) Effects of KRG only on PC12 cell. (c) Effects of KRG on 5% alcohol-treated PC12 cells. Cells were seeded into a 96-well tissue-culture plate (1×10^4 cells/ml). KRG was treated with or without various concentrations. After 6 hr of incubation, 5% alcohol was added for 24 hr, and 10 μl of Ez-Cytox reagent was added to each well. The 96-well plates were read with an ELISA microplate reader (Molecular Device, Sunnyvale, CA, USA) at 450 nm. Data are expressed as means ± S.D. (n=6). #p<0.05, ###p<0.001 compared to basal cells. *p<0.05 compared to alcohol-treated control cells. CTL; control, KRG; Rg3-enriched Korean red ginseng.

3.2 Effects of KRG on Bcl−2, Bid, Bax, and caspase−3 levels in alcohol−treated PC12 cell

Alcohol is known to induce neuronal cell apoptosis. Several studies indicated that KRG suppressed cell death. When PC12 cells were treated with alcohol for 24 hr, the level of the anti-apoptosis protein Bcl-2 significantly was reduced, by 58% compared with the control. In contrast, pre-treatment with 0.5 μg/ml, 1 μg/ml of KRG was significantly increased to 137%, 158% in a dose-dependent manner (Fig. 2). In addition, 0.5 μg/ml, 1 μg/ml of KRG reduced the level of apoptosis protein Bid, Bax, caspase-3 on the alcohol-treated PC12 cells. When PC12 cells were

treated with alcohol for 24 hr, the level of the apotosis protein Bid, Bax, caspase-3 were increased with compared to those of the control (171, 123 and 179%, respectively). In contrast, pre-treatment with 0.5 μg/ml, 1 μg/ml KRG was significantly reduced in a dose-dependent manner (Fig. 2).
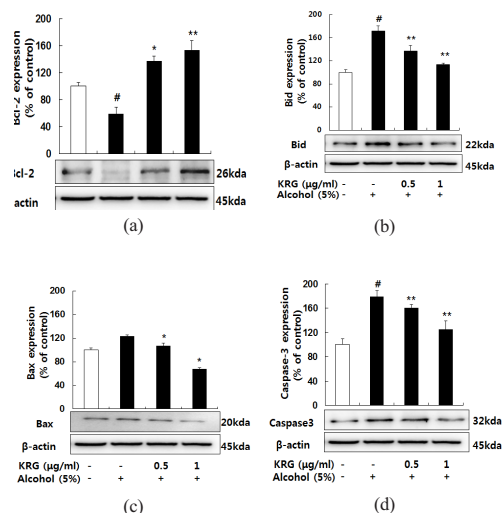

(a)

(b)

(c)

(d)

Fig. 2. Effects of KRG on Bcl-2, Bid, Bax, and caspase-3 levels induced by alcohol. (a) Effect of KRG on Bcl-2 activity. (b) Effect of KRG on Bid activity. (c) Effect of KRG on Bax activity. (d) Effect of KRG on caspase-3 activity. PC12 cells were pre-incubated with or without 0.5 and 1 μg/ml KRG for 6 hr, and then treated with or without 5% alcohol for 24 hr. Data are expressed as the means ± S.D. #p<0.05 compared to basal cells. *p<0.05, **p<0.001 compared to alcohol-treated control cells. CTL; control, KRG; Rg3-enriched Korean red ginseng.

3.3 Effects of KRG on apoptosis in alcohol−treated PC12 cell

As illustrated in Fig. 3, the percentage of apoptotic cells increased to 7.54% when annexin V + PI loaded cells (10^3 cells/test) were treated by 5% alcohol. However, this was significantly reduced by various concentrations (0.5 and 1 μg/ml) of KRG in a dose-dependent manner (4.18 and 2.87%, respectively). The inhibition rate by KRG (0.5 and 1 μg/ml) was 44.6 and 61.9%, respectively. These results suggest that the

anti-apoptotic activity of KRG on alcohol-treated PC12 cells was due to lowering of apoptotic cell.
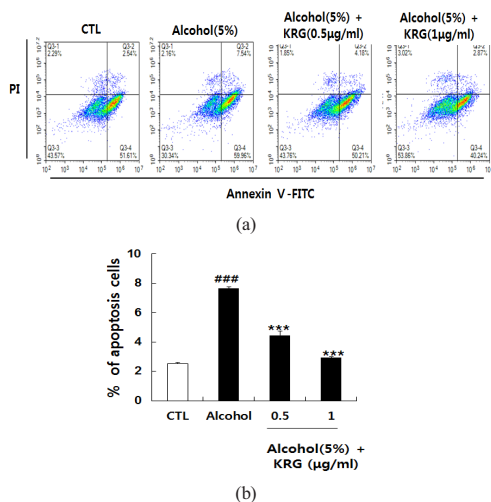

(a)

(b)

Fig. 3. Effects of KRG on alcohol-treated apoptosis in PC12 cells. (a) FACS detection using double staining of annexin V and prodium iodide (PI). (b) Quantification of apoptosis rate based on FACS results. Annexin V + PI loaded cells (10^3 cells/test) were preincubated with or without KRG (0.5 and 1 μg/ml), and then 5% alcohol was added. Data are expressed as means ± S.D. (*n*=3). ###*p*<0.001 compared to basal cells. ***p*<0.001 compared to alcohol-treated control cells. CTL; control, KRG; Rg3-enriched Korean red ginseng.

4. Discussion

PC12 cells are derived from rat adrenal pheochromocytoma, which is similar to neurons, and are often replaced by neurons in experiments that cause damage by substances such as MPP + and alcohol [18-20]. Alcohol is a small organic molecule that is widespread and complicated in the nervous system [20]. One of the main principles that cause damage to the central nervous system to kill neurons is to cause an increase in reactive stress species (ROS), mitochondrial dysfunction and DNA damage, resulting in neuronal cell death and necrosis [21]. Recent studies have shown that many natural drugs extracted from plants play an important role in protecting nerve cells from damage. Among them, KRG has been reported to have a neuroprotective effect against oxidative stress. Therefore, it can have a beneficial effect on alcohol-induced neurotoxicity. However, the effect of KRG on alcohol-induced nerve damage is ambiguous and the molecular mechanism has not yet been elucidated.

In order to investigate the protective effect of KRG on alcohol-induced apoptosis in PC12 cells, we performed a preliminary experiment to induce cell death in PC12 cells using 5% ethanol in this study, and the cell viability and the optimal dose of KRG were experimented, and cell apoptosis was measured. The expression of Bcl-2, Bid, Bax, and caspase-3 was analyzed to further clarify possible mechanisms. The results show significant cell death in PC12 cells treated with 5% alcohol and a decrease in cell viability. All these results are in agreement with previous studies [21-23] and demonstrate that the model is successful. On the other hand, treatment with KRG showed an inhibitory effect on alcohol-induced cell death of PC12 cells. Concentration of KRG 1 μg/ml showed the most remarkable protective effect on alcohol induced-cell death in PC12 cells. Mitochondrial dysfunction or oxidative stress plays an important role in alcohol-induced neuronal damage mechanisms, and mitochondrial membrane potential loss is one of the key factors for neuronal cell death [17,21]. Therefore, if KRG inhibits apoptosis of PC12 cells, it can be expected to be the result of protection of the mitochondrial membrane potential loss. In addition, mitochondrial apoptotic pathway is considered to be one of major pathways of apoptosis. In general, active oxygen free radicals and other factors are believed to cause mitochondrial damage to mitochondrial membrane potential reduction, nuclear membrane rupture and cytochrome C release, activation of cytochrome C-activated caspase-9 and the key enzyme caspase-3. As a result of this experiment, it was shown that caspase-3 expression in alcohol-damaged PC12

cells was increased, whereas KRG treatment effectively suppressed caspase-3 expression.

In addition, Bcl-2 family members play an important role in cell death [24,25]. Bcl-2 can inhibit apoptosis, modulating the redox state of mitochondrial thiol to regulate membrane potential and cell death. When apoptosis occurs, mitochondrial thiol can form a sensor of intracellular redox potential. Bcl-2 inhibits glutathione (GSH) leaking and lowers cytoplasmic redox potential to inhibit apoptosis. Bcl-2 inhibits the mitochondrial membrane potential and caspase-3 expression by controlling the membrane potential through regulation of the mitochondrial redox state of the thiol group, thereby further suppressing the loss. In addition, when apoptosis occurs via the extrinsic death receptor pathway, caspase-8 is activated, activating the BH3-only protein Bid, which is one of the Bcl-2 family members. When Bid is activated, it enters the intrinsic mitochondrial pathway and activates Bax, a 2-family protein, so that it can induce mitochondrial outer membrane permeabilization, which leads to further apoptosis. Therefore, when confirming the apoptosis process by caspase-3 expression, it is effective to confirm the change of Bid and Bax thtat are anti-apoptotic Bcl-2 family, and Bcl-2 that is apototic Bcl-2 family [26,27].

In the present study, KRG suppressed caspase-3 expression by increasing Bcl-2 expression and inhibiting Bid and Bax expression, which inhibited cell apoptosis. These results suggest that KRG-mediated up-regulation of Bcl-2 and down- regulation of Bid and Bax suppress caspase-3 expression, and ultimately inhibit the mitochondrial apoptotic pathway. This appears to be a protective mechanism of KRG on the apoptosis of PC12 induced by alcohol. This data is from in vitro experiments and it is necessary to further investigate the neuroprotective effects of KRG on ethanol-induced neurotoxicity and the chronic treatment of KRG in rodent models and humans. Nevertheless, this study provides novel evidence for the application of KRG as a candidate for neuroprotection.

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